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Development of a high-performance capillary isoelectric focusing technique with application to studies of microheterogeneity in chicken conalbumin

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Abstract

A robust, simple, reproducible isoelectric focusing method using capillary electrophoresis that exhibits high stability, migration time reproducibility and pH linearity over a wide pH gradient was developed. Consecutive runs (over 113 runs) of several proteins and one peptide with isoelectric points (p*I*s) ranging from 9.45 to 2.75 yielded excellent migration time reproducibility (<2% R.S.D.). Experimental parameters including buffer aging and capillary-to-capillary variation were thoroughly examined and optimized to improve the migration time reproducibility. The capillary isoelectric focusing (CIEF) method was applied to the analysis of chicken conalbumin (ovotransferrin), an iron-binding protein in egg white. Conalbumin (low iron content) separated into three major components with p*I*s of 7.2, 6.6 and 6.2. When the protein was saturated with iron (2 Fe/mol), a shift to lower p*I*s was detected. Chicken serum transferrin subjected to CIEF gave a pattern similar to conalbumin with three p*I*s of 7.1, 6.6 and 6.1, indicating that it was not fully saturated with iron. Thus, CIEF can be used as a potential analytical method to provide information about the metal-binding properties of specific metalloproteins.

Keywords: Conalbumin; Metalloproteins

1. Introduction

Isoelectric focusing (IEF) has been widely used for separation of proteins based on differences in their isoelectric points. The IEF method is accomplished by electrophoresis of proteins or peptides through a stable pH gradient until they reach the pH equal to their isoelectric point (p*I*), at which point the net charge and electrophoretic mobility are zero. The method has long been used to separate, isolate, purify and analyze a variety of proteins. The recent development of capillary electrophoresis (CE) led to the performing of IEF in a capillary format. Using 10–50 μm diameter capillary with capillary isoelectric focusing (CIEF), Joule heat generated by the electrophoretic process can be reduced to a minimum, resulting in the elimination of convection effects and a highly efficient separation. The use of a narrow diameter capillary also permits analysis with minute sample volume requirements. In addition, the UV transparency of the fused-silica allows on-column detection with CIEF, avoiding the necessity of

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the time-consuming steps of staining, destaining, densitometry, etc. The on-column detection also makes possible the applicability of IEF to the analysis of peptides, that could not otherwise be detected by staining.

CIEF was first introduced by Hjérten and co-workers [1–4]. The method involved focusing the proteins in the capillary and subsequently mobilizing the focused protein zones past the on-column detector. The mobilization step was accomplished either by hydrodynamically pumping the zones past the UV monitor (with voltage applied to avoid zonal broadening during elution), or by electrophoretic elution, achieved by replacing the catholyte or anolyte with a salt solution, which caused the pH gradient to move in the capillary. Recently, at least two different mechanisms have been proposed to mobilize the focused zones for detection [5–10]. Mazzeo and Krull [5,6] and Thormann et al. [7,8], independently demonstrated that electroosmotic flow (EOF) can be utilized to mobilize the focused protein zones. Chen and Wiktorowitz [9] showed that mobilization can be achieved by applying a vacuum to pull the focused zones through the separation capillary. Alternately, Huang et al. [10] used a pressure-driven mobilization scheme allowing the achievement of linearity of the pH gradient, high resolution and accuracy and precision of p*I* determination. A CIEF technique without mobilization was demonstrated by Wu and Pawliszyn [11,12], using an on-line CCD imaging detection system to replace on-column UV detectors.

Since the CIEF separations are performed in a capillary with its ends immersed in two vials filled with buffers of extreme pHs, the stability and reproducibility of the CIEF method has been problematic. This has hindered its acceptance as a routine analytical tool by the CE community. To become a rugged alternative to the slab gel method, a CIEF method needs to fulfill the requirements for reliability, reproducibility and long-term stability. The latter two parameters are especially important when the CIEF method is used in conjunction with automated instruments to become a fully robotic analytical tool for performing consecutive separations with high sample throughput and minimal labor requirements. In this study, we have improved the previous pressure-driven CIEF method [10]. An extensive evaluation of the method in terms of pH linearity, migration time reproducibility and long-term stability are reported. The applications of the CIEF method to analytical biochemistry are demonstrated by the study of microheterogeneity in chicken conalbumin (ovotransferrin) and transferrin, two iron-binding proteins present in egg white and chicken serum, respectively.

2. Experimental

2.1. Materials

In this study, ampholytes, poly(ethylene oxide) solution (with trade name of cIEF gel), a neutral coated capillary of 50 μm I.D., and four p*I* markers: cholecystikinin (CCK) flanking peptide (p*I* 2.75), β-lactoglobulin A (bovine milk, p*I* 5.1), carbonic anhydrase II (human erythrocytes, p*I* 5.9), and ribonuclease A (bovine pancreas, p*I* 9.45) from eCAP cIEF 3–10 kit (Beckman, Fullerton, CA, USA) were used. The neutral, hydrophilic coating inside the capillary wall consists of covalently bonded layers, which can effectively reduce the electroosmotic flow (EOF) in the capillary to 0.05–0.5×10^{-4} $cm^2/V\,s$ at pH 2–10. EOF has been shown to distort the linearity of the pH gradient with CIEF separations in a non-coated capillary [10]. The other protein p*I* markers and chicken conalbumin (egg white, iron-free and iron-complexed) were from Sigma Chemical (St. Louis, MO, USA). Chicken serum transferrin was from Cappel (Durham, NC, USA).

2.2. Capillary isoelectric focusing

P/ACE 2210 and 5510 capillary electrophoresis instruments controlled by System Gold software (Beckman) were used in this study. The total length of capillary used was 27 cm, with a separation length of 20 cm. The separations were carried out at 20°C with detection at 280 nm.

The capillary was prepared for a CIEF run as illustrated in Fig. 1. First the capillary was filled with a mixture of ampholytes and samples, by applying the high-pressure rinse mode (20 psi) for 1 min. Then, a voltage of 13.5 kV (500 V/cm) was applied

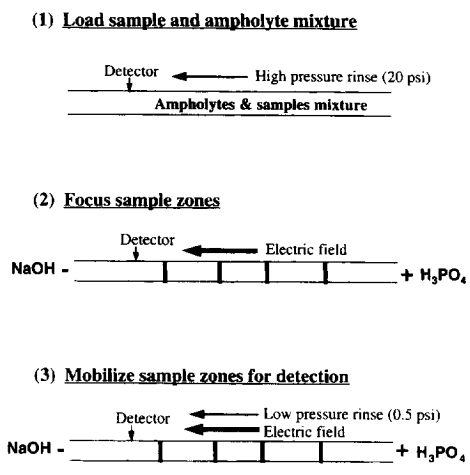

Fig. 1. Schematic diagram of the procedure for capillary isoelectric focusing (CIEF) with combined voltage and pressure-driven mobilization.

for 2 min to focus the ampholytes and samples. Since the applicable pH range of the ampholytes was extended, a section of ampholytes with p*I* values higher than 10 will be focused beyond the detector during the focusing step, forcing the samples with p*I* range of 3–10 to be focused before the detection window (see Fig. 1). The use of ampholytes with extended pH ranges as in this method eliminates the extra steps of filling the capillary with the correct amounts of electrode buffers and ampholytes to ensure the detection of samples [9,10]. Furthermore, since the sample was introduced into the capillary by filling the whole capillary with a mixture of sample and ampholyte solution, the injected sample amount can be easily and accurately controlled by adjusting the concentration of sample in the mixture. At the end of 2 min of focusing the system was programmed to apply a low-pressure rinse mode (0.5 psi) to mobilize the focused zones past the detector window, while applying a high voltage (13.5 kV) simultaneously to maintain the pH gradient for focusing the sample zones in the presence of the distorting effects of hydrodynamically induced laminar flow. The catholyte was an aqueous solution of 20 m*M* NaOH and the anolyte was 91 m*M* phosphoric acid in the cIEF gel solution. Between runs, the column was rinsed with 10 m*M* phosphoric acid for 1 min, followed by deionized water for 2 min.

3. Results and discussion

3.1. Applicable pH range and linearity

The ability to form a wide and stable pH range is essential to an IEF method intended for quality control, screening samples for impurities, and analysis of samples with extreme p*I* values (>9 or <4). It is well known that the conventional slab gel system has problems with the stability of pH gradients at extreme pHs [13,14]. The limitation of pH range also applies to other CIEF methods using chemical [4] or electroosmotic mobilization [4,7]. For the former method, two separations with different mobilization schemes (cathodic and anodic) have to be performed consecutively to obtain a wide pH gradient for scanning samples. For the latter, samples with p*I* values less than 4 can not be detected due to the elimination of electroosmotic flow at low pH. On the other hand, regardless of the chemistry status of samples, capillary or electrolyte buffers, hydrodynamic mobilization will drive the focused sample zones past the detector with the same speed, leading to the possibility of quantitative and qualitative analysis of samples in a wide pH range [9,15].

As shown in Fig. 2(a), standards with p*I* values ranging from 9.45 to 2.75 can be separated in one run in order of decreasing p*I*. This emphasizes the usefulness of the combined pressure–voltage CIEF method to wide-pH screening. The linearity of the pH gradient was determined by plotting the p*I* vs. retention time as shown in Fig. 2(b). The p*I*s of CCK-flanking peptide and ribonuclease A were derived from a previous literature citation [9]. Fig. 2(b) demonstrates that the CIEF method can provide a linear pH range at least between pH 2.75 and 9.45 (CCK-flanking peptide and ribonuclease A, respectively). The linearity of the pH gradient would ensure accuracy of p*I* estimation especially when the CIEF method is used for analysis of samples with unknown p*I*s [10].

3.2. Effects of capillary stability and buffer aging on migration time reproducibility

The long-term stability of the CIEF method was tested with four p*I* markers ranging from 2.75 to 9.45, and the results are shown in Table 1. The

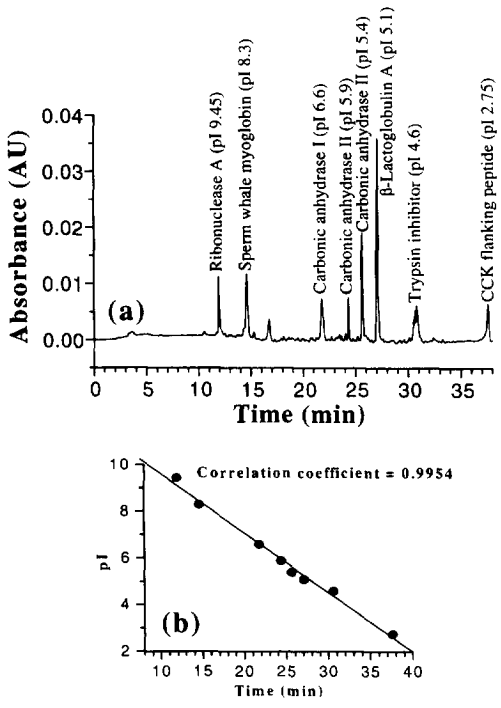

Fig. 2. (a) CIEF electropherogram of p*I* markers in a neutral coated capillary. (b) Linearity of the pH gradient measured by plotting the p*I* of markers against retention time. The p*I* markers were introduced into a separation capillary by filling the whole capillary with an ampholytes–markers mixture using the high-pressure rinse mode (20 psi) for 1 min. Concentrations of markers in the ampholytes–markers mixture: ribonuclease A (106 μg/ml), sperm whale myoglobin (69 μg/ml), carbonic anhydrase I (17.7 μg/ml), carbonic anhydrase II (p*I* 5.9, 6.6 μg/ml), carbonic anhydrase II (p*I* 5.4, 13.3 μg/ml), β-lactoglobulin A (53.1 μg/ml), trypsin inhibitor (26.5 μg/ml), CCK-flanking peptide (13.3 μg/ml).

relative standard deviations (R.S.D.s) for each of the markers for 113 runs were all less than 2%. The excellent migration time reproducibility can be attributed to the stability of the coated capillary and the addition of polymeric solution into sample and electrolyte buffer vials. In addition to its use as a viscosity-enhancing agent in order to reduce disturbance of focused zones caused by the hydrodynamic flow profile [10], it was found in our studies that the polyethylene oxide also serves as a dynamic coating to help the stability of the covalently bonded layer in coated capillaries. The uses of polymeric additives for deactivation of fused-silica capillaries to improve reproducibility of migration time have been reported in the literature [16,17].

Table 1
Migration time reproducibility of p*I* markers for consecutive CIEF separations

Standard	p*I*	Migration time[a]	
		Average	R.S.D.
Ribonuclease A	9.45	9.8	1.4
Carbonic anhydrase II	5.9	20.6	1.9
β-Lactoglobulin A	5.1	22.6	1.9
CCK-flanking peptide	2.75	31.2	1.8

[a] Based on 1st–113th runs (n=113).

A detailed study of the migration time pattern of the p*I* markers was carried out with the vials replaced after every 20 runs, and the results are shown in Fig. 3. Generally, the migration time of each marker tended to increase slightly with the number of runs until the vials were replaced. Furthermore, as shown in Fig. 3, the average values of the migration time for the first 20 runs were not significantly different from those of the last 13 runs, demonstrating the stability of the capillary during the long-term consecutive CIEF separations (75 h).

When the CIEF method is to be used for routine

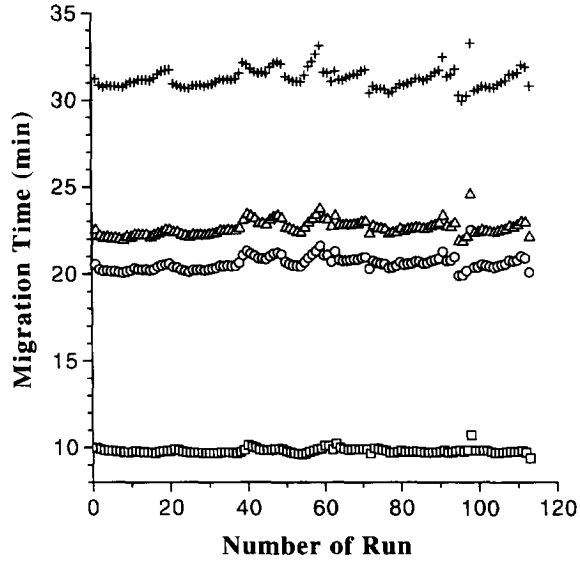

Fig. 3. Migration times of 113 consecutive runs for CIEF of p*I* markers. (□) Ribonuclease A, (○) carbonic anhydrase II, (△) β-lactoglobulin A, (+) CCK flanking peptide. The concentrations of markers in the ampholytes–markers mixture were the same as Fig. 4.

analysis of diverse biological samples, it is preferable that the electrolyte buffer vials, including catholyte, anolyte and rinsing buffer, be used as long as possible. On the other hand, electrolyte buffers are subject to aging during the electrophoresis process leading to variability in migration times [18]. In preliminary experiments, the effect of buffer aging on the migration time reproducibility was examined. When the sample vials (200 μl) and buffer vials (4 ml) were replaced after every 28 runs, overall migration time reproducibility (for 110 runs) increased (3% R.S.D.). The major cause of the aging effect was found to be evaporation of the polymeric solution in the sample and anolyte vials causing an increase in viscosity of the sample solution and anolyte.

Since the solution viscosity directly affects the migration time of analytes in a capillary under the application of pressure-driven mobilization, the separation speed can thus be manipulated by changing the solution. As shown in Fig. 4, by diluting the cIEF gel to 75% of the original concentration, the total separation time of the standards can be decreased to less than 23 min, while maintaining excellent resolution and long-term stability. The R.S.D.s of the standards were all less than 4%, based on the calculation of the first 20 runs and the last 20 runs ($n=40$) of 234 consecutive separations (Table 2). The use of a polymeric solution with lower viscosity, however, would adversely affect the separation resolution of closely migrating species such as hemoglobin variants A and F (p*I* difference of ca. 0.05, data not shown).

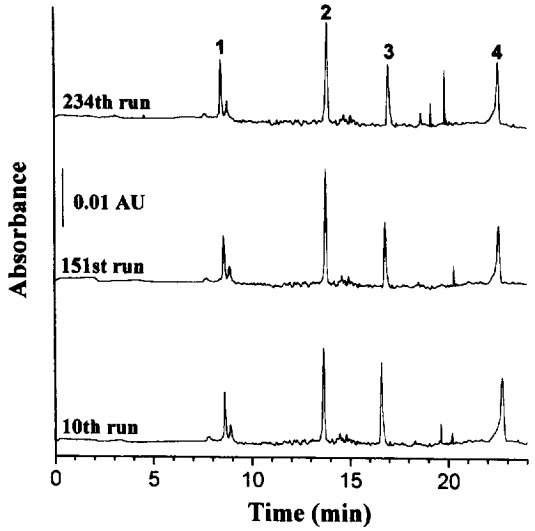

Fig. 4. Consecutive CIEF separations of p*I* markers, as listed in Table 2, with diluted cIEF gel. Compared to Fig. 2 and Fig. 3, the separation time was decreased by diluting the polymer solution to 75% of the original concentration. Concentrations of markers in the ampholytes–markers mixture: ribonuclease A (338 μg/ml), carbonic anhydrase II (29.2 μg/ml), β-lactoglobulin A (56.3 μg/ml), CCK-flanking peptide (28.2 μg/ml).

3.3. Capillary-to-capillary variation of migration time reproducibility

In this study, it has been found that the capillary-to-capillary variation in migration time is predominantly affected by the consistency of the inner diameter (I.D.) of the fused-silica capillaries. Since the hydrodynamic force driving the fluid in a capillary is proportional to the cross sectional area of the capillary, the migration time is thus inversely proportional to the square of I.D. of the capillary. Assuming that the coating layer does not significantly reduce the I.D. of a coated capillary, a migration time difference of 17% is expected for two capillaries with an I.D. difference of 4 μm. Experimentally, a migration time deviation of 15% has been observed among the coated capillaries made from different lots (the normal manufacturing tolerance of inner diameter for the 50 μm I.D. bare fused-silica capillary is ±3 μm) [19]. To improve capillary-to-capillary variation in migration time reproducibility, custom-made fused-silica capillaries with an I.D. tolerance of ±1 μm were used. Migration time reproducibility was examined using different lots of the custom-

Table 2
Migration time reproducibility of p*I* markers for consecutive CIEF separations with diluted cIEF gel[a]

Peak No.	Standard	Migration time [b]	
		Average	R.S.D.
1	Ribonuclease A	8.5	2.1
2	Carbonic anhydrase II	13.5	2.4
3	β-Lactoglobulin A	16.5	2.5
4	CCK-flanking peptide	22.3	3.4

[a] The CIEF gel was diluted to 75% of the original concentration before use.
[b] Based on 1st–20th runs and 215th–234th runs ($n=40$).

Table 3
Capillary-to-capillary variation of migration time reproducibility[a]

Standard	Migration time[b]	
	Average	R.S.D.
Ribonuclease A	9.6	2.1
Carbonic anhydrase II	21.8	3.6
β-Lactoglobulin A	23.7	3.6
CCK-flanking peptide	33.6	3.1

[a] Based on four different capillary lots, of which one capillary was randomly selected from each lot.
[b] Five separation runs were performed for each capillary, ($n=4\times 5=20$).

made coated silica capillaries. As shown in Table 3, the R.S.D.s can then be reduced to below 4%. It is worth while to mention that variation in ID between capillaries might also affect the migration time reproducibility among capillaries with other modes of capillary electrophoresis (especially capillary gel electrophoresis), due to the dependence of buffer viscosity on the Joule heat, which is proportional to the cross-sectional area of capillary.

3.4. Analysis of chicken conalbumin

Metalloproteins play many important roles in processes that are basic to cellular function such as respiration, metabolism and gene expression. Metals bound to these proteins serve either as catalytic or structural cofactors. In order to fully characterize a particular metalloprotein, it is necessary to explore the binding status of the metal(s) to the protein. Different modes of capillary electrophoresis have been applied to the study of a variety of metalloproteins, including metalloenzymes, heme-containing proteins, plasma metal-transporting proteins and intracellular metal storage proteins [20]. In this study, the capillary isoelectric focusing was applied to the analysis of chicken conalbumin (ovotransferrin), an iron-binding protein present in egg white, and chicken serum transferrin, an iron-binding protein present in chicken blood. As shown in Fig. 5(a), conalbumin (low iron) separated into three major components with p*I*s of 7.2, 6.6 and 6.2. The p*I*s of component peaks shown were derived by linear regression analysis of three internal p*I* markers denoted by number (1, 2, 3) above the peaks. These

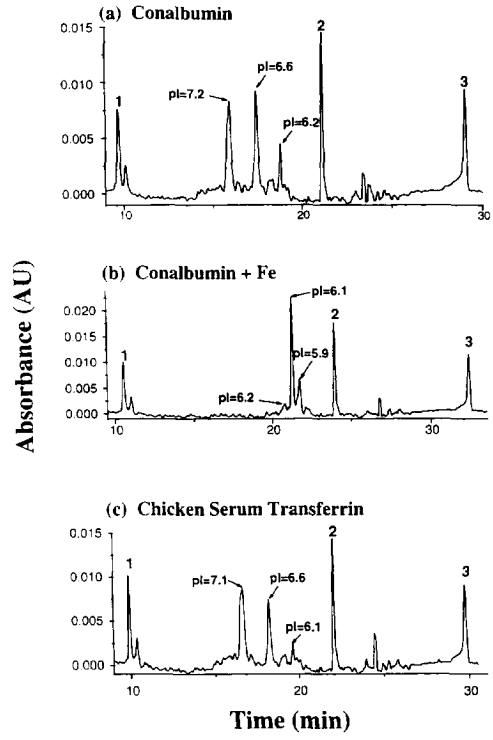

Fig. 5. Separations of (a) chicken egg conalbumin, (b) conalbumin saturated with iron and (c) chicken serum transferrin by CIEF. The p*I*s of component peaks are shown and were derived by linear regression analysis of three internal p*I* markers denoted by number (1, 2, 3) above the peaks. 1=ribonuclease A, 2=β-lactoglobulin A, 3=CCK flanking peptide. Concentrations of proteins and markers in the ampholytes–sample mixture: ribonuclease A (338 μg/ml), β-lactoglobulin A (84.5 μg/ml), CCK-flanking peptide (28.2 μg/ml), conalbumin (46.7 μg/ml), iron-saturated conalbumin (46.7 μg/ml), chicken serum transferrin (62.1 μg/ml).

three peaks may constitute the different metalloforms of conalbumin including: the iron-free (p*I* 7.2), the monoferric (p*I* 6.6), and the diferric (p*I* 6.2) forms described previously [21]. Saturation of conalbumin with iron (2 Fe/mol protein) prior to CIEF resulted in a shift of the peaks to more acidic pHs (Fig. 5(b)), as has been reported previously for both conalbumin [21] and human serum transferrin [22–25]. A predominant peak at p*I* 6.1 (presumably the diferric form) and two minor peaks at p*I*s of 6.2 and 5.9 were resolved from the iron-saturated conalbumin sample. The two minor peaks would arise due to microheterogeneity in the attached carbohydrate, since conalbumin is a glycoprotein [21]. The putative iron-

free form (p*I* 7.2 in Fig. 5(a)) was absent from the iron-saturated sample.

Chicken serum transferrin, a protein with a structure very similar to chicken egg conalbumin, when subjected to CIEF gave a pattern similar to that observed for conalbumin with peaks corresponding to the iron-free form (p*I* 7.1), the monoferric form (p*I* 6.6) and a small amount of the diferric form (p*I* 6.1); see Fig. 5(a). Judging from the similarity of CIEF separation pattern and p*I* values of component peaks between chicken egg conalbumin and chicken serum transferrin (Fig. 5(a) and Fig. 5(c)), we conclude that transferrin apparently circulates in chicken blood in a relatively unsaturated state with the largest fraction being the iron-free form.

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